Molecular cloning, characterization and regulation of two different NADH-glutamate synthase cDNAs in bean nodules

LOURDES BLANCO¹, PALLAVOLU M. REDDY¹, SONIA SILVENTE¹, BRUNA BUCCIARELLI³, SANGHAMITRA KHANDUAL¹, XOCHITL ALVARADO-AFFANTRANGER², FEDERICO SÁNCHEZ², SUSAN MILLER³, CARROLL VANCE³ & MIGUEL LARA-FLORES¹

¹Centro de Ciencias Genómicas and ²Instituto de Biotecnología, Universidad Nacional Autónoma de México, Av Universidad, C.P. 62210, Cuernavaca, Morelos, México and ³Agronomy and Plant Genetics, University of Minnesota and United States Department of Agriculture/Agriculture Research Service, Plant Science Research Unit, 1991 Upper Buford Circle, St Paul, MN 55108, USA

ABSTRACT

NADH-dependent glutamate synthase (NADH-GOGAT) is a key enzyme in primary ammonia assimilation in Phaseolus vulgaris nodules. Two different types of cDNA clones of PvNADH-GOGAT were isolated from the nodule cDNA libraries. The full-length cDNA clones of PvNADH-GOGAT-I (7.4 kb) and PvNADH-GOGAT-II (7.0 kb), which displayed an 83% homology between them, were isolated using cDNA library screening, 'cDNA library walking' and RT-PCR amplification. Southern analysis employing specific 5' cDNA probes derived from PvNADH-GOGAT-I and PvNADH-GOGAT-II indicated the existence of a single copy of each gene in the bean genome. Both these proteins contain ~100 amino acid sequences theoretically addressing each isoenzyme to different subcellular compartments. RT-PCR analysis indicated that PvNADH-GOGAT-II expression is higher than PvNADH-GOGAT-I during nodule development. Expression analysis by RT-PCR also revealed that both of these genes are differentially regulated by sucrose. On the other hand, the expression of PvNADH-GOGAT-I, but not PvNADH-GOGAT-II, was inhibited with nitrogen compounds. In situ hybridization and promoter expression analyses demonstrated that the NADH-GOGAT-I and -II genes are differentially expressed in bean root and nodule tissues. In silico analyses of the NADH-GOGAT promoters revealed the presence of potential cis elements in them that could mediate differential tissue-specific, and sugar and amino acid responsive expression of these genes.

Key-words: Phaseolus vulgaris; carbon and nitrogen compounds; gene expression; promoter analysis; PvNADH-GOGAT.

Correspondence: M. Lara-Flores. Fax: +52 777 317 5581; e-mail: lara@ccg.unam.mx

INTRODUCTION

Glutamine synthetase (GS) and glutamate synthase (GOGAT) form the main route of ammonia assimilation in higher plants (GS/GOGAT cycle) (Miflin & Lea 1976; Lancien, Gadal & Hodges 2000; Suzuki & Knaff 2005). GS catalyses the ATP-dependent amidation of glutamate resulting in the production of glutamine. GOGAT, on the other hand, catalyses the reductive transfer of the amide group of glutamine to the α -keto position of 2-oxoglutarate, forming two molecules of glutamate (Lea, Robinson & Stewart 1990). Ammonia in higher plants is derived both from the primary nitrogen sources (ammonia, nitrate and for legumes, dinitrogen) and from a number of internal nitrogen-cycling pathways such as photorespiration, amino acid metabolism and phenylpropanoid metabolism (Vance 1997). GOGAT is therefore essential not only for primary nitrogen assimilation but also to maintain the general nitrogen economy of the plant.

In higher plants, GOGAT occurs as two distinct isoforms: Fd-dependent GOGAT (Fd-GOGAT; EC 1.2.7.1) and NADH-dependent GOGAT (NADH-GOGAT; EC 1.4.1.14), and both isoforms differ from one another in molecular mass, subunit composition, enzyme kinetics, reductant specificity and metabolic function (Temple, Vance & Gantt 1998; Lea & Miflin 2003). These two molecular species of GOGAT exist in both green and non-green tissues. The Fd-GOGAT has been shown to be the most abundant isoform in green tissues and is located in the chloroplast (Wallsgrove, Lea & Miflin 1979). This monomeric enzyme is an Fe-S protein with an M_r about 150 kDa. In leaves, Fd-GOGAT catalyses the assimilation of NH₄+ derived from both the light-dependent reduction of NO₃⁻ and the NH₄⁺ generated during photorespiration, while the root isoform has been implicated in the assimilation of NH₄⁺ derived from soil NO₃⁻ (Sakakibara et al. 1991; Redinbaugh & Campbell 1993; Suzuki & Knaff 2005). Complimentary DNA of Fd-GOGAT has been isolated from a number of species. Two Fd-GOGAT genes (GLU1 and GLU2) were characterized in Arabidopsis thaliana; GLU1

plays a major role in the assimilation of ammonium derived from photorespiration, whereas the GLU2 gene may play a major role in the primary nitrogen assimilation in roots (Oliveira et al. 1997; Suzuki & Rothstein 1997; Coschigano et al. 1998).

NADH-GOGAT is found primarily in the nonphotosynthetic tissues of plants, such as roots and legume root nodules, where it is located in the plastids. In Medicago sativa, it has been immunolocalized to the amyloplasts of infected cells in the nodules (Trepp et al. 1999a). Pattern of expression was found to shift during root nodule development and expression is tightly linked to effective nodules (Trepp et al. 1999b). It has been purified from legume root nodules as well as rice (Oryza sativa) cell cultures (Chen & Cullimore 1988; Anderson et al. 1989; Hayakawa et al. 1992). As Fd-GOGAT, NADH-GOGAT is an Fe-S flavoprotein (Curti et al. 1995; Suzuki & Knaff 2005). NADH-GOGAT exists as a monomer with an M_r of approximately 225–230 kDa (Yamaya et al. 1992; Gregerson et al. 1993; Vance et al. 1995). In nodules, it is the major form of GOGAT, and where it combines with cytosolic GS to assimilate NH₄+ produced by the nitrogenfixing bacteroids. NADH-GOGAT activity has been found to increase markedly during nodule development (Gregerson et al. 1993; Vance et al. 1994), and is three times higher than the Fd-GOGAT activity. It has been proposed that NADH-GOGAT catalyses the rate-limiting step in ammonia assimilation in the root nodules (Trepp et al. 1999a,b).

In non-legumes such as rice, NADH-GOGAT has been characterized with studies of gene organization, expression of mRNA, protein localization and protein structure (Yamaya et al. 1992, 1995; Goto et al. 1998). The expression of the NADH-GOGAT gene in rice is regulated in a cell type-specific, age-specific and nitrogen-responsive manner (Hayakawa et al. 1994, 1999). It has been located in roots, leaves, etiolated leaves, vascular bundles of unexpanded leaf tissues and the developing grains, and was shown to function in the primary assimilation of NH₄⁺ derived from soil NO₃⁻ and the re-assimilation of NH₄⁺ released during amino acid catabolism and/or during seed germination.

Full-length cDNA and genomic clones of NADH-GOGAT have been isolated from M. sativa and O. sativa, and was found that NADH-GOGAT is encoded by a single gene (Gregerson et al. 1993; Vance et al. 1995; Goto et al. 1998). The only report that described the existence of two isoforms of NADH-GOGAT in plants is in bean nodules where two isoenzymes (I and II) were identified (Chen & Cullimore 1988). The isoenzyme NADH-GOGAT-II is localized in the central tissue of the nodule, while the isoenzyme NADH-GOGAT-I is found to be present both in the cortex and central tissue of the nodules, as well as in the roots (Chen & Cullimore 1989). NADH-GOGAT-II accounted for the majority of the activity in the root nodules. Specific activity of NADH-GOGAT in roots is found to be about 27 times lower than that in the nodules, and consisted almost entirely of NADH-GOGAT-I. Both isoenzymes were partially purified, and were shown to have

similar M_rs of about 200 kDa with marginal differences in kinetic characteristics (Chen & Cullimore 1988).

In this paper, we report for the first time the isolation, characterization, localization and regulation of expression of two distinct types of cDNAs encoding NADH-GOGAT from bean (Phaseolus vulgaris ev. Negro Jamapa) nodules. The data presented here indicate that the two NADH-GOGAT isoforms described by Chen & Cullimore (1988) in the *P. vulgaris* nodules are encoded by two distinct genes. The expression analysis suggests different roles for these two NADH-GOGAT isoforms in the assimilation of ammonia in symbiotically nitrogen-fixing bean plants.

MATERIALS AND METHODS

Plant growth, treatments and tissue sampling

P. vulgaris cv. Negro Jamapa seeds (ProNaSe, Guanajuato, México) were surface sterilized in 20% sodium hypochlorite solution for 10 min, washed thoroughly with sterile distilled water and germinated on a moist sterile filter paper. Threeday-old seedlings were transferred to the pots containing sterile vermiculite (five plants per pot) and then inoculated with Rhizobium tropici CIAT899, Rhizobium etli CE3 or R. etli CE3 NifA-. The uninoculated plants served as the control. The plants were grown in a naturally lighted greenhouse as described by Ortega et al. (1992). For the PvNADH-GOGAT expression analysis, the nodules were harvested at various time points during 12-21 d after inoculation (DAI). In addition, roots, stems and fully expanded leaves of 13-day-old nodulated plants, and cotyledonary leaves from 3-day-old plants, and flowers and pods from mature plants were also collected for analysis. In the studies pertaining to assessing the effects of carbon and nitrogen treatments on the PvNADH-GOGAT expression, the plants were watered for 4 d, starting on the 17th DAI, with the Hoagland nutrient solution containing either sucrose (0.5, 1 and 2%), asparagine, glutamine, xanthine, allantoin (10 mM) or allopurinol (2 mM), and then the nodules were harvested for analysis. The nodules were also collected from the control plants that were treated with the Hoagland nutrient solution devoid of any carbon and nitrogen compounds. All tissues were immediately frozen in liquid nitrogen and stored at -70 °C until use.

Isolation of RNA

Poly(A)+ RNA for constructing the cDNA library was isolated from the nodules following the standard method (Sambrook, Fritsch & Maniatis 1989). Total and poly(A)⁺ nodule RNA for isolating full-length bean NADH-GOGATs by RT-PCR were extracted using RNeasy Plant Kit and Oligotex mRNA Mini Kit (Qiagen, Valencia, CA, USA), respectively. The total RNA from the plant tissues for the expression analysis by RT-PCR was isolated by employing the RNA Tripure Isolation Reagent (Roche Diagnostics Corporation, Indianapolis, IN, USA).

Isolation of PvNADH-GOGAT cDNAs

Two λ ZAP cDNA libraries (constructed using λ ZAPcDNA synthesis Kit; Stratagene, La Jolla, CA, USA) of P. vulgaris derived from poly(A)+ RNA of 15-day-old nodules were screened for isolating PvNADH-GOGAT clones. The cDNA probes were labelled with ³²P using random primer labeling kit (Megaprime DNA labelling system; Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK), and employed to screen the cDNA libraries. The library screening was done on nylon membrane (Amersham Life Sciences, Little Chalfont, UK) replicas by standard procedures (Sambrook et al. 1989). Hybridization was carried out overnight in 5X Denhardt's solution (1X Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 6X SSPE (1X SSPE is 0.15 M NaCl, 10 mM sodium phosphate and 1 mM ethylenediaminetetraacetic acid, pH 7.4), 1% sodium dodecyl sulphate (SDS) and 0.1 mg mL⁻¹ denatured salmon sperm DNA at 55 °C. Membrane filters were washed once at room temperature for 5 min in 2X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and 0.1% SDS, and then twice at 50 °C for 20 min each in 0.2X SSC and 0.1% SDS. Positive λ ZAP clones were plaque purified and cDNA inserts were obtained in the Bluescript SK-vector by in vivo excision according to the manufacturer's instructions (Stratagene).

Library screening was done in two steps. Initial library screening was done using a 1.6 kb cDNA probe derived from the alfalfa NADH-GOGAT (pGOGAT7.2; Gregerson et al. 1993). As a consequence, we isolated two clones containing cDNA inserts of 2.5 kb (PvNADH-GOGAT-I) and 3 kb (PvNADH-GOGAT-II). Subsequently, utilizing these partial PvNADH-GOGAT-I and PvNADH-GOGAT-II cDNAs as probes, longer cDNA inserts were obtained by screening a second nodule cDNA library, which was prepared using size-fractionated (3-9 kb) nodule cDNA. From the second screening, two longer clones of 5 kb each corresponding to PvNADH-GOGAT-I and -II were isolated and sequenced. In order to expand the 5' region of the cDNA inserts, size-fractionated nodule cDNA library was used as a template for 'cDNA walking', and three sequential rounds of PCR (profile A; Supplemental Table S1) using Expand Long Template PCR System (Roche Diagnostics Corporation) were performed using pBluescript T3 primer in combination with gene specific 5' region primers designed for each of the PvNADH-GOGAT cDNA insert/fragment (gene-specific reverse primers for the amplification of 5' regions; Supplemental Table S2). The gene specific 5' primers were derived from the sequence analysis of the cDNA inserts in the clones as well as the amplified fragments obtained from each round of PCR amplification.

Finally, RT-PCR was performed in order to obtain the full-length clones for each of the *PvNADH-GOGATs*. For this purpose, fresh nodule cDNA was synthesized from the total RNA isolated from 15-day-old nodules using Power-Script reverse transcriptase (Clontech, Palo Alto, CA, USA), primed with oligo (dT)₁₂₋₁₈ (Invitrogen, Carlsbad,

CA, USA), and employed for the amplification of the full-length cDNAs using gene-specific 5' and 3' primers (for amplifying full-length clones; Supplemental Table S2) designed based on the assembled sequences of the clones obtained through both library screening and 'cDNA library walking' (see above). In the PCR step of the reaction, the Expand Long Template PCR System (Roche Diagnostics Corporation) was used for the amplification of the full-length 7.4 kb *PvNADH-GOGAT-II* and 7.05 kb *PvNADH-GOGAT-II* (profile B; Supplemental Table S1). Both fragments were cloned in pGEM-T easy vector (Promega Corp., Madison, WI, USA) and transformed into XL10 GOLD *Escherichia coli* cells (Stratagene) and sequenced.

Sequence analysis

DNA sequencing of all isolated clones was done at Medigenomix (Munich, Germany), and the sequences of the full-length clones were corroborated with those sequences previously obtained from the partial clones. To manage the sequence data, the DNA strider 1.3 program (Marck 1988) was used. Similarity searches of databases were performed according to Altschul et al. (1990) with the NCBI BLAST server (http://www.ncbi.nih.gov/BLAST/). Transit peptide was identified and analysed with Plant-Ploc (Plant Protein Subcellular Location Prediction; http:// 202.120.37.186/bioinf/plant/; Chou 2005; Shen & Chou 2006; Chou & Shen 2007) and WoLF PSORT (Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences; http://wolfpsort.org/; Horton et al. 2006, 2007) programmes. The protein molecular masses and pI were estimated by using the pI/MW program at Uniprot (EMBL-EBI; http://au.expasy.org/tools/pi_tool.html).

RT-PCR analysis

The tissue samples were ground in liquid nitrogen, and the total RNA was extracted with Tripure (Roche Diagnostics Corporation) according to the instructions of the manufacturer. For RT-PCR studies, 5 μ g of total RNA was converted into first-strand cDNA by using the PowerScript preamplification system (Clontech) and oligo (dT)₁₂₋₁₈ (Invitrogen). One microlitre of cDNA sample was amplified (profile C; Supplemental Table S1) in a 50 μ L PCR mixture containing DNA polymerase buffer (Roche Diagnostics Corporation), 2.5 mM MgCl₂, 200 μ M of each dNTPs, 0.1 μ M of each gene-specific primers (RT-PCR primers; Supplemental Table S2) and 1 unit of Taq DNA polymerase (Roche Diagnostics Corporation).

Plant DNA extraction and DNA gel blot analysis

Genomic DNA was isolated from the leaves of 5-day-old seedlings using the DNAzol procedure (Gibco BRL Life Technologies, Inc., Grand Island, NY, USA). For the gel blot analyses, genomic DNA was digested with PstI, XbaI, KpnI and EcoRV restriction enzymes, separated on a 0.8% agarose gel (15 μ g DNA per lane) and transferred to nylon

membranes (Hybond-N+, Amersham Life Sciences). Hybridization was performed using, as labelled probes, a 611 bp and a 461 bp fragments specific for PvNADH-GOGAT-I and PvNADH-GOGAT-II, respectively. Both fragments contain the 5' untranslated region (UTR) and transit peptide-coding regions. The probes were radio labelled with ³²P by random priming with rediprime II system (Amersham Life Sciences). Hybridization was carried out overnight at 65 °C in 300 mM phosphate buffer (pH 7.2) and 7% SDS. The hybridized filters were washed at 65 °C with 2X SSC (15 min), 0.2X SSC (15 min) and 0.1X SSC (10 min) plus 0.1% (w/v) SDS, and exposed to Kodak X Omat films.

Isolation of the NADH-GOGAT promoters

The 5' flanking genomic sequences of PvNADH-GOGAT-I and PvNADH-GOGAT-II were isolated by long-distance PCR using bean DNA and GenomeWalker kit (Clontech) according to the manufacturer's instructions. Gene specific primers employed for genome walking of the 5' flanking sequences of PvNADH-GOGAT-I and PvNADH-GOGAT-II are given in the Supplemental Table S3. Longdistance PCR was performed using Advantage 2 Polymerase Mix with the following cycling profiles. Primary PCR amplification was carried out with five initial cycles of 28 s at 94 °C and 4 min at 72 °C, followed by 20 cycles of 28 s at 94 °C and 4 min at 67 °C, and a single final cycle of 7 min at 67 °C. Secondary PCR was carried out with an initial cycle of 1 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 64 °C, and 2 min at 72 °C, and a single final cycle of 7 min at 72 °C. Isolated 5' flanking regions were sequenced at Medigenomics, and the transcription start sites were determined using the Neural Network Promoter Prediction Program (Reese 2001; http://www.bdgp.org/seq_tools/ promoter.html) in conjunction with the untranslated 5' sequences of PvNADH-GOGAT-I and PvNADH-GOGAT-II cDNAs. The promoter sequences were also explored for identifying cis-acting regulatory elements in them using PLACE (Higo et al. 1999; http:// www.dna.affrc.go.jp/PLACE/) as well as PlantCARE databases (Lescot et al. 2002; http://bioinformatics.psb.ugent. be/webtools/plantcare/html/).

Development of plant transformation vectors and agrobacterial strains carrying the binary vectors, and inoculum preparation

The binary vectors pBI-PrPvNADH-GOGAT-I-EGFPGUS and pBI-PrPvNADH-GOGAT-II-EGFPGUS containing the P. vulgaris NADH-GOGAT promoters transcriptionally coupled to the egfp-gusA coding fusion sequence and the 3' nos terminator sequence were constructed as follows. Five prime flanking sequences comprising of 1501 bp PvNADH-GOGAT-I and 1366 bp PvNADH-GOGAT-II, upstream of their respective coding sequences were PCR amplified as described above, digested with SalI and XmaI (restriction sites were embedded in the forward and reverse primers used for promoter amplification; see Supplemental Table S3) and cloned upstream of the egfp-gusA coding sequence in a similarly digested pBI101-EGFPGUS binary vector yielding pBI-PrPvNADH-GOGAT-I-EGFPGUS pBI-PrPvNADH-GOGAT-II-EGFPGUS. The pBI101-EGFPGUS vector was constructed by replacing gusA coding sequence in pBI101.1 (Clontech) with egfpgusA chimeric reporter gene derived from pHGWFS7 (Karimi, Inze & Depicker 2002).

The binary vectors used for the bean transformation, pBI101-EGFPGUS (promoterless EGFPGUS, control vector), pBI-PrPvNADH-GOGAT-I-EGFPGUS or pBI-PrPvNADH-GOGAT-II-EGFPGUS, were transformed into Agrobacterium rhizogenes K599 by electroporation (Nagel et al. 1990). A. rhizogenes strains harbouring the transformation vectors were grown at 30 °C in Luria-Bertani (LB) plates supplemented with 50 µg mL⁻¹ kanamycin. The bacterial cells from overnight-grown culture from a single plate were harvested and resuspended in 5 mL sterile distilled water, and used for infecting the bean seedlings for the induction of hairy root formation (see below).

Hairy root transformation and arowth conditions

Bean seeds harvested from the greenhouse-grown plants were surface sterilized and germinated in pots containing sterile vermiculite in the plant growth room (maintained at a 14 h light/10 h dark cycle and a temperature of 25 °C) as described earlier (Silvente et al. 2008), and irrigated with the Summerfield nutrient solution (Summerfield, Huxley & Minchin 1977) supplemented with 5 mM KNO₃. The hairy root transformation of the bean plants was performed according to Estrada-Navarrete et al. (2006) as modified by Silvente et al. (2008) using A. rhizogenes strains carrying the binary vectors pBI101-EGFPGUS (control vector), pBI-PrPvNADH-GOGAT-I-EGFPGUS or pBI-PrPvNADH-GOGAT-II-EGFPGUS. A. rhizogenes-transformed composite bean plants were grown in pots and inoculated with 1-2 mL R. tropici CIAT899 culture (rhizobial inoculum was prepared by growing CIAT899 strain to a density of 10⁶ cells mL⁻¹ in peptone-yeast extract (PY) medium supplemented with nalidixic acid, 20 μg mL⁻¹), irrigated with the Summerfield nutrient solution (Summerfield et al. 1977) having a highly reduced level (0.385 mM) of KNO₃ and incubated in the plant growth chamber maintained at a 14 h light/10 h dark cycle and a temperature of 25 °C. After 4-5 d of acclimatization, the transformed plants were transferred to the naturally lit greenhouse maintained at 25 °C. The plants (five replicates per each treatment) were harvested 3-4 weeks after rhizobial inoculation, and the roots and nodules were analysed for GUS and GFP activity. GUS and GFP analysis revealed no conspicuous plant to plant variations in the pattern or level of expression of the reporter genes.

Histochemical localization of GUS activity and other microscopic methods for the visualization of GFP

GUS staining of the transgenic roots was performed at 30 °C for 12–24 h essentially as described by Reddy *et al.* (1998). After staining, the roots were rinsed in 100 mM phosphate buffer and fixed for more than 4 h in a solution containing 3.7% formaldehyde, 5% acetic acid and 50% ethanol, examined as whole specimens and photographed under a stereomicroscope (Carl Zeiss Stemi 2000-C; Carl Zeiss Jena GmbH, Jena, Germany).

For the GFP analysis, the roots and nodules were processed essentially according to Estrada-Navarrete et al. (2006). Briefly, the tissues were cross-linked with 100 uM m-maleimidobenzoyl-*N*-hydroxysuccinimide-ester, with 4% paraformaldehyde, passed through dehydration ethanol-methacrylate series, and finally embedded by polymerization in butyl-methacrylate/methyl-methacrylate (4:1, vol/vol; Sigma Aldrich, St Louis, MO, USA) mixture with 0.375% (wt/vol) benzoin ethyl ether (Fluka Chemika, Buchs, Switzerland) in gelatin capsules (Electron Microscopy Sciences, Fort Washington, PA, USA). The embedded tissue was sectioned (3 µm thickness) using ultramicrotome (Leica, Bensheim, Germany), processed to remove methacrylate, mounted with citifluor (Ted Pella, Inc., Redding, CA, USA) and observed for GFP fluorescence using a Zeiss LSM 510 Meta confocal microscope equipped with Axiovert 200M. GFP excitation was obtained at 488 nm using an argon laser and an HFT UV 488/543/633-nm dual dichroic excitation mirror, and the fluorescence was detected with an LP 505-nm emission filter. The images were processed using Adobe Photoshop Elements 2.0 software (Adobe Systems Incorporated, Mountain View, CA, USA).

In situ hybridization

All RNA probes were generated from linearized pBluescript plasmids containing either the 602 bp *PvNADH-GOGAT-II* fragment, the 563 bp *PvNADH-GOGAT-III* fragment or the 600 bp *Pv*-leghemoglobin fragment. Both *PvNADH-GOGAT-II* (between 6695 and 7297 bp) and *II* (between 6419 and 6982 bp) fragments (probes) were derived from the non-homologous 3' UTR and C terminal coding regions of the full-length cDNA clones. The plasmids harbouring *PvNADH-GOGAT-I*, -*II* or *Pv*-leghemoglobin fragments were linearized with the appropriate restriction enzymes and transcribed in the sense and antisense directions using the T3 or T7 RNA polymerase and radio labelled with ³⁵S UTP nucleotide. The hydrolysis of the transcribed probes and the subsequent *in situ* hybridization protocols have been described by Trepp *et al.* (1999b).

The sequence data from this paper have been deposited at the Genbank under the accession numbers *PvNADH-GOGAT-II* cDNA, AF314925; *PvNADH-GOGAT-II* cDNA, AF314924; *PvNADH-GOGAT-II* promoter, AB195997; and *PvNADH-GOGAT-II* promoter, AB195998.

RESULTS

Isolation and characterization of two bean cDNAs encoding NADH-GOGAT

In order to isolate NADH-GOGAT homologs from the bean (P. vulgaris), we first screened a 15-day-old root nodule cDNA library by hybridization with a 1.6 kb cDNA probe derived from M. sativa NADH-GOGAT. Consequently, two clones containing bean cDNA inserts of 2.5 kb (PvNADH-GOGAT-I) and 3 kb (PvNADH-GOGAT-II) were isolated. The sequencing of both cDNA clones revealed an open reading frame (ORF) encoding a partial polypeptide similar to MsNADH-GOGAT, with different 3' UTR sequences between them. The detection of two cDNAs of PvNADH-GOGAT with different 3' UTR sequences suggested the presence of two PvNADH-GOGAT genes in the bean. RNA gel blot analysis utilizing specific PvNADH-GOGAT clones as probes revealed 7 kb mRNAs as expected for proteins with a molecular mass higher than 200 kD. In order to obtain longer PvNADH-GOGAT cDNA clones, 2.5 and 3 kb fragments of PvNADH-GOGAT-II and PvNADH-GOGAT-II, respectively, were used as hybridization probes to screen a second bean nodule cDNA library, which was prepared with sizefractionated (3-9 kb) nodule cDNA. From the second screening, two longer clones of 5 kb each corresponding to PvNADH-GOGAT-I and PvNADH-GOGAT-II were isolated. Full-length PvNADH-GOGAT-I (7406 bp) and PvNADH-GOGAT-II (7050 bp) cDNA clones were subsequently obtained by 'cDNA library walking' with nested amplification by PCR using the bean root nodule library as template (see material and methods). Sequence analysis of different sub-clones, as well as of RT-PCR derived fulllength cDNA clones, confirmed the existence of two different PvNADH-GOGAT genes in bean.

The sequence of the PvNADH-GOGAT-I cDNA (7406 bp) showed that it contains a 435 bp 5' UTR with several translation stop codons in all three reading frames before the first ATG, and revealed an ORF 6591 bp long, starting at residue 436. A 367 bp long 3' UTR and a 13 bp polyA+ were also defined. PvNADH-GOGAT-I ORF codes for a 2196 amino acid polypeptide with a predicted molecular mass of 241 kD and a theoretical pI of 6.1 (Fig. 1). PvNADH-GOGAT-II clone is 7050 bp long with a 5' UTR of 189 bp with several translational stop codons in all three reading frames before the first ATG codon start site. The PvNADH-GOGAT-II clone showed a single ORF of 6582 bp from 190 to 6771 bp, a 3' UTR 264 bp long and 15 bp polyA⁺. Putatively, the PvNADH-GOGAT-II codes for a 2192 amino acid polypeptide with a predicted molecular mass of 241 kD and a pI of 6.18 (Fig. 1). Complete coding regions of both clones showed 81% identity at the nucleotide level and 86% at the amino acid level. It is important to note that the theoretical pI predicted for the PvNADH-GOGAT deduced proteins of the present study closely correlated with the pI 6.069 and 6.159, respectively, reported for PvNADH-GOGAT-I and -II earlier (Chen & Cullimore 1988).

A BLAST search of the protein databases with the deduced amino acid sequences of PvNADH-GOGAT-I and PvNADH-GOGAT-II showed an identity of 85 and 83%, respectively, with the MsNADH-GOGAT (Fig. 1). Additionally, PvNADH-GOGAT-I and -II polypeptides showed an identity of 78% with A. thaliana and 74% with O. sativa NADH-GOGATs. PvNADH-GOGAT-I and -II, at amino acid level, showed an identity ranging from 39 to 46% with Fd-GOGAT from A. thaliana (GLU1 and GLU2), Spinacia oleracea, Zea mays, O. sativa and Glycine max.

Functional domain analysis

Deduced amino acid sequences indicated that each of the PvNADH-GOGAT I and PvNADH-GOGAT-II contain a pre-sequence of 98 and 96 amino acids, respectively, resembling that of MsNADH-GOGAT (Gregerson et al. 1993). The analysis using Plant-Ploc and WoLF PSORT programmes suggested a plastid targeting sequence for both PvNADH-GOGAT-I and PvNADH-GOGAT-II. It is extraordinary that these pre-sequences are rich in Ser and Thr residues, but reduced in Asp, Glu and Tyr content, which is characteristic of the nuclear-encoded plastid precursor protein (Karlin-Neumann & Tobin 1986; von Heijne, Steppuhn & Herrmann 1989).

In addition, the examination of the predicted polypeptide sequences of the bean NADH-GOGAT isoforms revealed three main domains that are putatively responsible for various enzymatic functions as delineated in NADH-GOGATs of alfalfa and other plants: The first domain is concerned with carrying out the glutamine amido transferase reaction (Figs 1 & 2). Interestingly, within this domain, PvNADH-GOGAT-I exhibited a 26-amino acid sequence, which is different from both PvNADH-GOGAT-II and MsNADH-GOGAT (designated in bold letters in Fig. 1 and black square in Fig. 2). The second domain (underlined in Fig. 1) is involved in the binding of FMN, and located in close proximity to three cysteine residues (indicated by diamonds in Fig. 1) putatively implicated as a binding site for [3Fe-4S] cluster. FMN and [3Fe-4S] carry out the electron transport to the intermediate 2-iminoglutarate to form glutamate from 2-oxoglutarate. The third domain is an NADH-binding domain (double underlined in Fig. 1) and contains five conserved residues (identified by arrowheads in Fig. 1) that are important for the binding of the cofactor (Gregerson et al. 1993).

Genomic organization of NADH-GOGATs in the bean

In order to confirm if the two PvNADH-GOGAT clones represent two different genes and to assess whether there may be other homologs of these two NADH-GOGATs in the bean, genomic DNA Southern blot analysis was performed. As is shown in Fig. 3, a single hybridizing fragment was detected in each of the genomic DNA digestions tested. When a 611 bp probe containing 435 bp of the 5' UTR and 176 bp of the N-terminal coding region was used for the specific detection of PvNADH-GOGAT-I, a single hybridization fragment was detected in each of the DNA digestions tested (Fig. 3a). On the other hand, when a specific probe for the detection of PvNADH-GOGAT-II (461 bp long fragment containing 189 bp of the 5' UTR and 272 bp of the N-terminal coding region) was used, KpnI and EcoRV digestions generated each a single hybridizing fragment of the same size (Fig. 3b). PstI and XbaI digestions also generated each a unique hybridizing fragment of almost the same size but larger than those fragments generated by KpnI and EcoRV digestions (Fig. 3b). These results indicated that PvNADH-GOGAT-I and PvNADH-GOGAT-II represent two different NADH-GOGAT genes and that there is only a single copy of each gene in the P. vulgaris genome.

Expression of NADH-GOGATs in bean tissues

To determine whether the two P. vulgaris NADH-GOGATs are differentially expressed in various plant tissues, RT-PCR analysis was performed using RNA isolated from roots, stem, young cotyledonary leaves, mature leaves, flowers, pods and nodules. As is shown in Fig. 4, transcripts of both PvNADH-GOGATs were detected in all tissues analysed. In most tissues, excepting mature leaves, PvNADH-GOGAT-II expression was found to be higher than that of PvNADH-GOGAT-I (Fig. 4a,b). RT-PCR analysis utilizing mRNA isolated from the nodules harvested from the plants 12-21 d after rhizobial inoculation showed high and nearly constant levels of expression of PvNADH-GOGAT-II during all stages of nodule development as compared to PvNADH-GOGAT-I (Fig. 4a,b). In contrast, PvNADH-GOGAT-I showed a peak in expression only at day 18 following inoculation and a decline thereafter. These results indicated that both NADH-GOGAT genes are differentially regulated in various bean tissues including the nodules.

Relationship between PvNADH-GOGAT expression and nitrogen fixation

As already mentioned, NADH-GOGAT plays an important role in the ammonia assimilation pathway. In order to delineate the extent of the participation of individual PvNADH-GOGATs in the assimilation of the ammonia derived from the symbiotically nitrogen-fixing rhizobial cells, we analysed the expression of PvNADH-GOGAT-I and -II in fix+ and fix- nodules induced respectively by the CE3 wild type and the NifA- mutant strains of R. etli. RT-PCR analysis demonstrated that PvNADH-GOGAT-I expression was significantly lowered in non-fixing nodules (Fig. 4a,b). In contrast, the level of PvNADH-GOGAT-II expression remained unaffected in fix nodules, until at least 18 dpi, as compared to fix⁺ nodules. As these fix⁻ nodules

M.sat	MSNSLSLTFT	ALNNPQINAI	SNPN*ARLRP	LARVTRCSAT	TKLF.A CVERKRWLGT *.RVTKF	KLRSGG***G	LERIQLWESG	${\tt GLGRLPKLRV}$	AVKSSFSAVP		100
M.sat	FDKDSCGVGF	VAELNGQSSR	KTVTDALEML	VRMTHRGACG	CEANTGDGAG	ILVALPHGFY	QEVVDFQLPP	QGNYAVGMFF	LPKSDSRRKE		200
M.sat	${\tt SLGHKVLGWR}$	SVPTDNTGLG	KSAQLTEPVI	EQVFLTPSSD	R SKVDLEKQMY	ILRKLSMVSI	TSALNLQSDG	ITDFYICSLS	SRTVIYKGQL		300
M.sat	ADLGNERFTS	YMALIHSRFS	TNTFPSWDRA	QPFRV*LGHN	GEINTLRGNV	NWIKAREGLL	KCKELGLSEN	DLKKFLPIVD	ANSSDSGCFD	GVLEFLLHSG	400
M.sat	KSLPEAVMMM	IPEAWQNDKN	MDPQRKAFYE	YYSALMEPWD	GPALISFTDG	HYLGATLDRN	GLRPGRFYVT	HSGRVIMASE	VGVVDIPPED		500
M.sat	${\tt MMLLVDFEKQ}$	IVVNDDALKE	QYSLARPYGD	WLEKQKIELK	VPK.G DIIDSVHESD VQQ.E	IVPPTISGVP	PLSNDDVDME	${\tt NMGIQGLLAP}$	LKAFGYSVES	LEILLLPMAK	600
M.sat	DGVEALGSMG	NDTPLAVMSN	REKLTFEYFK	QMFAQVTNPP	IDPIREKIVT	SMRCMVGPEG	DLTETTEEQC	HRLSLKGPLL	STKEMEAIKK	MNYRGWRSKV	700
M.sat	IDITYSKERG	TKGLEEALDR	ICTEAHNAIS	EGYTTLVLSD	RAFSKKHVAV	SSLLAVGAVH	QHLVKTLERT	RVALMVESAE	PREVHHFCTL	VGFGADAICP	800
M.sat	YLAIEAIWRL	QVDGKIPPKA	SGDFNSKDEL	VKKYFKASTY	GMMKVLAKMG	ISTLASYKGA	QIFEALGLSS	EVIEKCFAGT	PSRVEGATFE	MLAQDALHLH	900
M.sat	ELAFPSRIFS	PGSAEAVALP	NPGDYHWRKG	GEVHLNDPLA	IAKLQEAART	NSVDAYKQYS	KTIHELNKAC	NLRGLLKFKD	AASKVPISEV		1000
M.sat	CTGAMSYGSI	SLEAHTALAT	AMNTIGGKSN	TGEGGEQPSR	PN.TM. MEPLADGSRNSM.	PKRSAIKQVA	SGRFGVSSYY	LTNADELQIK	${\tt MAQGAKPGEG}$	GELPGHKVIG	1100
M.sat	DIAITRNSTA	GVGLISPPPH	HDIYSIEDLA	QLIHDLKNAN	.T PAARISVKLV	SEAGVGVIAS	${\tt GVVKGHAEHV}$	LISGHDGGTG	ASRWTGIKSA	GLPWELGLAE	1200
M.sat	${\tt THQTLVANDL}$	RGRTTLQTDG	QLKTGRDVAI	AALLGAEEYG	FSTAPLITLG	CIMMRKCHKN	TCPVGIATQD	PVLREKFAGE	PEHVINFFFM	VAEEMREIMS	1300
M.sat	QLGFRTVNEM	VGRSDMLEVD	KEVVKGNAKL	ENIDLSLLLR	PAAELRPEAA	QYCVQKQDHG	LDMALDNKLI	SLSNAALEKG	LPVYIETPIC	NTNRAVGTML	1400
M.sat	SHEVTKRYNL	AGLPADTIHI	QFTGSAGQSF	GAFLCPGITL	ELEGDSNDYI	GKGLSGGKVV	VYPPKGSNFD	PKDNILIGNV	ALYGATRGEA	YFNGMAAERF	1500
M.sat	CVRNSGALAV	VEGVGDHGCE	${\tt YMTGGTVVVL}$	${\tt GKTGRNFAAG}$	MSGGIAYVLD	VDGTFQSRCN	LELVDLDKVE	EEEDIITLRM	LIQQHQRHTN	SLLAKEVLVD	1600
M.sat	${\tt FENLLPKFVK}$	VFPREYKRVL	ASMKSDAASK	DAVERAAEDV	ED DEQDDEAQAV E*V.MEL.	EKDAFEELKK	LATASLNEKP	SEA**PKRPS	QVTDAVKHRG	FVAYEREGVQ	1700
M.sat	YRDPNVRLND	WNEVMMETKP	GPLLKTQSAR	${\tt CMDCGTPFCH}$	QENSGCPLGN	KIPEFNELVY	QNRWQEALER	LLETNNFPEF	TGRVCPAPCE		1800
M.sat	PVSIKNIECA	IIDKAFEEGW	MIPRPPVKRT	GKRVAIVGSG	PSGLAAADQL	NKMGHIVTVF	ERADRIGGLM	${\tt MYGVPNMKTD}$	KVDIVQRRVN	LMAEEGINFV	1900
M.sat	VNANIGLDPL	YSLERLREEN	DAIVLAVGAT	KPRDLPVPGR	ELSGVHFAME	FLHANTKSLL	DSNLQDGNYI	SAKGKKVVVI	GGGDTGTDCI		SS 2000
M.sat	VVNLELLPQP	PPTRAPGNPW	PQWPRIFRVD	YGHQEAETKF	GKDPRTYEVL	${\tt TKRFVGDENG}$	VVKGLEVVRV	CWEKDETGKF	QFKEIEGSEE		2100
M.sat	${\tt MGFLGPEATI}$	AEKLGVERDN	RSNFKADYGR	FSTSVDGVFA	AGDCRRGQSL	VVWAISEGRQ	AAAQVDSYLT	${\tt NED*HGIDGN}$	QDEFVKRQQD	LNKKH***SK	2200
PvG.I M.sat PvGII	HTVMT										

Figure 1. Amino acid sequence alignment of bean PvNADH-dependent glutamate synthase (NADH-GOGAT)-I and PvNADH-GOGAT-II with alfalfa NADH-GOGAT. Residues numbering begins with the first amino acid in the proteins. Identical residues are indicated by dots. Asterisks denote the gaps introduced to maximize sequence similarity. Residues presented in italics represent targeting leader sequences. Residues represented in bold letters denote dissimilar/distinct amino acid sequence in the amido transferase domain of PvNADH-GOGAT-I. FMN- and NADH-binding domains are single and double underlined, respectively. Diamonds denote three cysteine residues involved in binding a [3Fe-4S] cluster. Five conserved residues that are important for NADH binding are indicated by arrowheads.

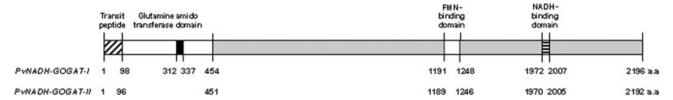


Figure 2. Diagramatic representation of the predicted PvNADH-dependent glutamate synthase (NADH-GOGAT)-I and PvNADH-GOGAT-II polypeptides showing different functional domains. Black box indicates 26 amino acid sequence that is different between these two polypeptides. Amino acid numbering starts with the initiation methionine in each polypeptide.

have a normal morphology and contain bacteroides in the infected cells, this result suggests that the expression of *PvNADH-GOGAT-II*, but not of *PvNADH-GOGAT-II*, is affected by the nitrogen status of the nodule and not is due by a developmental deficiency.

In an analogous study in *P. vulgaris*, working with fix⁺ and fix⁻ *Rhizobium* strains, Chen, Bennett & Cullimore (1990)

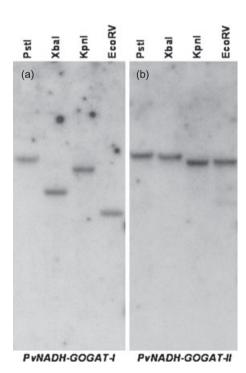


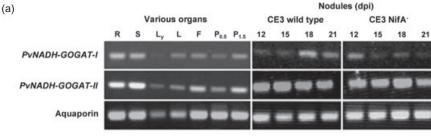
Figure 3. DNA gel blot analyses of PvNADH-dependent glutamate synthase (NADH-GOGAT)-I and PvNADH-GOGAT-II in bean genome. Bean DNA (15 μ g per lane) was digested with PstI, XbaI, KpnI or EcoRV, electophoresed through a 0.8% agarose gel, denatured, transferred to a nylon membrane and probed with (a) PvNADH-GOGAT-I (611 bp) and (b) PvNADH-GOGAT-II (461 bp) specific probes.

observed changes in the activity of NADH-GOGAT-I isoenzyme in the nodules in response to nitrogen fixation, while the induction of NADH-GOGAT-II isoenzyme remained unaltered. Based on these findings, it was concluded that unlike NADH-GOGAT-I, the induction of the NADH-GOGAT-II isoenzyme occurs independently of nitrogen fixation, but that nitrogen fixation was clearly required to increase and maintain the magnitude of the induction in *P. vulgaris*.

Regulation of *PvNADH-GOGAT* expression by carbon and nitrogen compounds

As the nitrogen status of the nodule affected the PvNADH-GOGAT-I but not PvNADH-GOGAT-II expression, we decided to study the effect of different nitrogen compounds as well as sucrose on the expression of these two NADH-GOGATs in nodules. RT-PCR analysis revealed that the transcript levels of PvNADH-GOGAT-I increased in the nodules derived from the plants treated with 0.5% sucrose, but no further enhancement was noticed when supplemented with higher sucrose concentrations (Fig. 5a,b). In contrast, the PvNADH-GOGAT-II expression increased concomitantly with the increase in sucrose concentration. The quantitative analysis of the sugars revealed that indeed the levels of metabolizable sugars are enhanced in the nodules of the plants supplemented with exogenous sucrose, fructose or glucose (Silvente et al. 2008). These findings, together with the above results, indicate that both PvNADH-GOGATs are positively regulated by carbon reserves, thus, implicating carbon availability as a limiting factor in the GS/GOGAT ammonia assimilation pathway in nodules. The presence of different nitrogenous compounds in the nutrient solution did not affect the expression of PvNADH-GOGAT-II in the nodules (Fig. 5a,b). However, PvNADH-GOGAT-I expression declined in the presence of amides (asparagine and glutamine), and most noticeably with ureides (xanthine and allantoine) and with allopurinol,

(b)



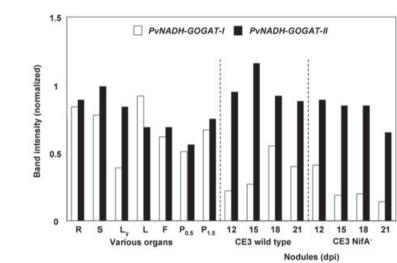


Figure 4. (a) RT-PCR analyses of bean PvNADH-dependent glutamate synthase (NADH-GOGAT)-I and PvNADH-GOGAT-II mRNA levels in various organs, and in developing nodules formed by Rhizobium etli CE3 wild type and NifA-mutant strains. Bean aquaporin expression is used as an internal reference. (b) Histogram depicting the ratios of the PvNADH-GOGAT-I and PvNADH-GOGAT-II band intensities (of the corresponding PCR products represented in 'a') normalized against the respective aquaporin bands. R, root; S, stem; L_v, young cotyledonary leaf; L, mature leaf; F, flower; P_{0.5}, pod of 0.5 cm long; P_{1.5}, pod of 1.5 cm long; dpi, days post inoculation.

which is an inhibitor of ureide synthesis. It is intriguing that both the ureides and the ureide inhibitor, like allopurinol, down regulate the expression of *PvNADH-GOGAT-I*. The inhibition of *PvNADH-GOGAT-I* by allopurinol probably suggests that the metabolites upstream of xanthine production (such as purine pathway intermediates) also negatively affect the *PvNADH-GOGAT-I* expression.

The above results indicate in one hand that both nodule *PvNADH-GOGAT*s are positively regulated by carbon availability and in the other, that nodule *PvNADH-GOGAT-I* is negatively regulated by metabolic intermediates of the ureide biosynthetic pathway.

Isolation and characterization of PvNADH-GOGAT-I and PvNADH-GOGAT-II promoters

To study how *PvNADH-GOGAT-I* and *PvNADH-GOGAT-II* genes are expressed in the roots and nodules, we isolated the promoter regions of these genes from the bean genomic DNA, employing the genome walking strategy, and utilized them to study their activities during root and nodule development in bean hairy root system (see materials and methods). The sequencing revealed that the cloned *PvNADH-GOGAT-II* and *PvNADH-GOGAT-II* promoters were 1501 and 1366 bp long, respectively. The determination of the transcriptional start sites in the promoters, employing the Neural Network Promoter Prediction Program (see materials and methods), enabled the precise delineation of a 436 bp long 5' untranslated region

(5' UTR) in *PvNADH-GOGAT-II* and a 192 bp long 5' UTR in *PvNADH-GOGAT-II* cDNA transcripts (Supplementary Fig. S1). A TATA box (TATATAT) was recognized in the promoter of *PvNADH-GOGAT-I* at positions –460 to –466 bp upstream of the translational initiation site (+1), whereas in the *PvNADH-GOGAT-II* promoter, the TATA box (TATAAA) was found to be at positions –215 to –220 bp. Putative CAAT box motifs were located at –930 to –933 bp and –254 to –257 bp, respectively, in the *PvNADH-GOGAT-II* and *PvNADH-GOGAT-II* promoters. Thus, the proximal regions of *PvNADH-GOGAT-II* and *PvNADH-GOGAT-II* promoters consist of the elements shown to be essential for the initiation of transcription in the promoters of other plants.

Tissue-specific expression patterns of PvNADH-GOGAT-I and PvNADH-GOGAT-II genes during root and nodule development

To elucidate the patterns of expression of *PvNADH-GOGAT-I* and *PvNADH-GOGAT-II* genes during root and nodule development, we have utilized *PvNADH-GOGAT* promoters fused to the *egfp-gusA* chimeric reporter gene (Fig. 6 top panel) and produced compound bean plants with transgenic hairy roots through *A. rhizogenes-*mediated transformation (see materials and methods). The hairy roots of the plants transformed with the control vector (promoterless *egfp-gusA* vector) served as the control. The transgenic hairy roots from the symbiotically grown control plants did not exhibit any GUS activity or GFP fluorescence

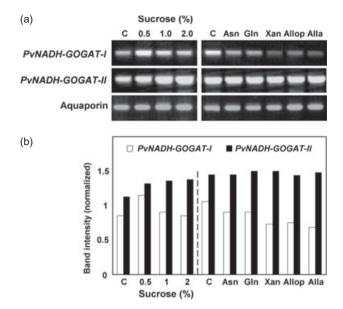


Figure 5. (a) RT-PCR analyses of bean PvNADH-dependent glutamate synthase (NADH-GOGAT)-I and PvNADH-GOGAT-II mRNA levels in the nodules of the bean plants treated with different carbon and nitrogen compounds. Bean aquaporin expression is used as an internal reference. Nodules nursed with (left panel) 0.5, 1.0 or 2.0% sucrose, and (right panel) 10 mM asparagine (Asn), glutamine (Gln), xanthine (Xan), allantoin (Alla) or 2 mM allopurinol (Allop). C, untreated nodules served as control. (b) Histogram depicting the ratios of the PvNADH-GOGAT-I and PvNADH-GOGAT-II band intensities (of the corresponding PCR products represented in 'a') normalized against the respective aquaporin bands.

in them. On the other hand, the transgenic roots of the plants transformed with the vectors carrying PvNADH-GOGAT-I and PvNADH-GOGAT-II promoter:egfp-gusA fusions displayed GUS/GFP expression in roots and nodules, though the intensity of expression varied depending on the promoter and developmental stage (Fig. 6). Both promoters exhibited an intense GUS expression in the meristematic cells located at the root tip (Fig. 6a,j). In the roots expressing the PvNADH-GOGAT-I promoter, intense GUS staining was observed in both young and mature portions of the root (Fig. 6a-f) as compared to the roots carrying the PvNADH-GOGAT-II promoter, which showed strong GUS expression mostly in the young portions of the roots (Fig. 6j) but not in the mature zones (Fig. 6k-m). In addition, the vascular tissues in the mature portions of the roots exhibited comparatively greater GUS expression with PrPvNADH-GOGAT-I than with PrPvNADH-GOGAT-II (compare Fig. 6b-d with k-m). The visualization of the GFP expression, however, revealed that the induction with both the promoters was relatively more pronounced in root vascular tissues vis-a-vis cortical cells (Fig. 6g,p). During the initiation of lateral root formation, the GUS expression with PvNADH-GOGAT-II promoter was relatively weaker in the meristematic cells situated in the lateral root primordia, as compared to the strong expression shown by the PvNADH-GOGAT-I promoter (compare Fig. 6b with k,l).

On the other hand, the histochemical staining of the roots transformed with both GOGAT promoters revealed relatively a strong GUS activity in the nodule primordia (Fig. 6c,d,m) as well as in the mature nodules (Fig. 6e,f,n,o). A detailed examination with confocal microscopy, as revealed by EGFP expression, established that the activity of both promoters was mainly restricted to the infected cortical cells in the central zone of the nodules, although more intensely with the PvNADH-GOGAT-II promoter than the PvNADH-GOGAT-I promoter (Fig. 6h,i,q,r). In contrast to the infected cortical cells, the uninfected cells virtually showed no EGFP expression. The outer cortical cells of the nodules, on the other hand, exhibited a low EGFP induction with both the promoters; here again in the outer cortical cells the expression with PrPvNADH-GOGAT-I being less intense than PrPvNADH-GOGAT-II.

In order to further verify the sites of expression of both PvNADH-GOGAT genes, and to precisely identify the locations of accumulation of PvNADH-GOGAT-I and PvNADH-GOGAT-II mRNA transcripts in the nodules, we performed in situ hybridization studies. For this, longitudinal sections of paraffin-embedded P. vulgaris mature root nodule tissues were hybridized with sense or antisense 35S UTP-labelled RNA probes (Fig. 7) for either PvNADH-GOGAT-I or -II. Results showed that both PvNADH-GOGAT-I and -II are expressed within the central tissue of the bean root nodule (Fig. 7a,d). A higher magnification of this region demonstrated that PvNADH-GOGAT-I and -II mRNAs are mostly localized within the infected cells (Fig. 7j,k). As expected, the nodule sections probed with the Pv-leghemoglobin transcript, used as a positive control, showed a high expression in the central tissue of the nodule (Fig. 7g), specifically, the infected cells. Thus, the in situ hybridization results have provided additional confirmation showing the infected cells as the major sites of expression of both NADH-GOGAT genes, as evidenced with the expression of respective promoters in the bean nodules (see above; Fig. 6).

In silico identification of the potential cis-acting elements that regulate tissue-specific, and sugar and amino acid responsive expression of the NADH-GOGAT promoters

Since PvNADH-GOGAT-II and PvNADH-GOGAT-II exhibited differential expression in root tissues (Fig. 6), we performed in silico analysis to assess whether any differences could be deciphered in these promoters with regard to the putative cis-elements that were shown to mediate tissuespecific expression in other plants. PLACE and PlantCARE database searches revealed the presence of a large array of putative cis-acting elements in both PvNADH-GOGAT-I (~95 motifs) and PvNADH-GOGAT-II (~70 motifs) promoters that potentially participate in the regulation of gene expression in response to various cellular and environmental cues. In both PvNADH-GOGAT promoters, we found cisacting elements that were shown to mediate tissue-specific expression in various other plants (Fig. 8a-c). Even though

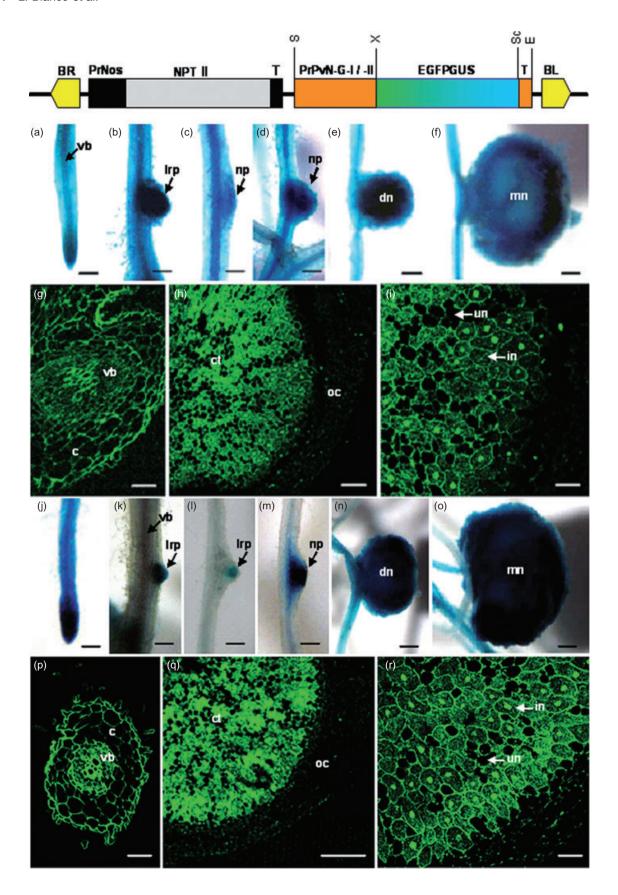


Figure 6. Top panel: basic structure of T-DNA in the binary vectors pBI-PrPvNADH-GOGAT-I-EGFPGUS and pBI-PrPvNADH-GOGAT-II-EGFPGUS used in *Agrobacterium rhizogenes* K599-mediated hairy root transformation of *Phaseolus vulgaris*. (a–r) Patterns of *PvNADH-GOGAT-I* (a–i) and *PvNADH-GOGAT-II* (j–r) promoter activity as visualized by GUS and EGFP expression in roots and nodules of *P. vulgaris*. Bright field stereo-micrographs showing histochemical staining of *PvNADH-GOGAT-I* and *PvNADH-GOGAT-II* promoter-mediated GUS expression in roots (a–d and j–m), lateral root primordia (b and k, l) and nodule primordia (c, d and m), and fully matured nodules (e, f and n, o). Laser confocal micrographs showing *PvNADH-GOGAT-I* (g–i) and *PvNADH-GOGAT-II* (p–r) promoter-mediated GFP expression in 3-μm-thick root (g and p) and nodule (h, i and q, r) sections. Bar length equals 50 μm (g, i, p, r), 100 μm (h), 200 μm (a, j, q), 250 μm (b, c, k–m), 300 μm (e, n), 350 μm (f, o), 400 μm (d). BR, right border; BL, left border; PrNos, nopaline synthase promoter; NPTII, kanamycin resistance gene; T, *nos* terminator; PrPvN-G-I / II, *PvNADH-GOGAT-I* or *II* promoter; EGFPGUS, *egfp-gusA* fusion gene; S, *SaI*I; X, *XbaI*; Sc, *SacI*; E, *Eco*RI; vb, vascular bundle; lrp, lateral root primordium; np, nodule primordium; dn, developing nodule; mn, mature nodule; c, cortex; ct, central tissue; oc, outer cortex; in, infected cell; un, uninfected cell.

both promoters harboured meristematic and vascular tissuespecific regulatory motifs, the PvNADH-GOGAT-I promoter contained more of them, both in terms of number and diversity, than PrPvNADH-GOGAT-II (Fig. 8a,b). In contrast, PvNADH-GOGAT-II contained a higher concentration of nodule-specific *cis*-elements in its promoter (Fig. 6c). In both promoters, we have also located several sugar responsive plant cis-acting elements including those responsible for sucrose-inducible expression (Fig. 8d). cis-Elements that regulate sugar repression were also detected in both promoters, though more of such motifs were found in PrPvNADH-GOGAT-I than in PrPvNADH-GOGAT-II (Fig. 8e). In addition, both promoters harboured consensus sequences of cis-elements that resembled amino acid responsive motifs encountered in mammalian asparagine synthetase and CHOP (homolog of CAAAT/enhancerbinding protein) gene promoters (Guerrini et al. 1993; Bruhat et al. 2000, 2002; Barbosa-Tessmann et al. 2000; Fig. 8f). Consensus sequences of the conserved cis-elements located in both NADH-GOGAT promoters that potentially mediate tissue-specific, and sugar and amino acid responsive expression, together with relevant references, are included in Fig. 8 and its legend. In addition to the above mentioned cis-elements, both the promoters also exhibited stretches of AT-rich regions and numerous perfect direct and invert repeats of varying lengths up to 14 bp (not shown). Future work can only decipher the functional significance of these repeat motifs.

DISCUSSION

Legume plants can obtain their nitrogen from atmospheric N_2 through symbiotic association with rhizobia. Ammonia, which is fixed by rhizobia, is assimilated in the plant via the joint action of GS and GOGAT, which convert glutamine and 2-oxoglutarate into glutamate. These two reactions are now generally agreed to be the primary route of nitrogen assimilation in plants, and the products glutamine and glutamate, donate their amido and amino groups for the synthesis of most of the nitrogen-containing compounds of the cell. One interesting point is that, in non-nodulated bean plants, the assimilated ammonia is mobilized in the form of amides, but when bean plants are symbiotically fixing nitrogen, ureides are the main nitrogen-containing compounds involved in the transport of the nitrogenase-derived

ammonia to the rest of the plant (Atkins 1982). To complement our understanding of the ammonia assimilation process in bean nodules, we isolated and characterized two NADH-GOGAT cDNAs, studied their expression in different plant tissues and assessed their regulation by carbon and nitrogen compounds in the root nodules. This research extends our understanding of legume root nodule nitrogen metabolism and NADH-GOGAT by: (1) demonstrating that NADH-GOGAT in bean is encoded by two separate genes having distinct patterns of expression; (2) the expression of the PvNADH-GOGAT-1 is reduced by products of nitrogen fixation; (3) in the 5'-upstream region of both PvNADH-GOGAT genes selected cis-elements are conserved between the two; (4) the 5'-upstream region of both GOGAT genes drives reporter gene expression to infected cells of root nodules; and (5) effective nitrogen fixation is not required for the expression of either PvNADH-GOGAT genes, suggesting that the regulation of the NADH-GOGAT genes in bean nodules may be under developmental control.

Two distinct clones of 7406 bp (PvNADH-GOGAT-I) and 7050 bp (PvNADH-GOGAT-II) cDNAs capable of encoding a 241 kD protein each, were isolated and characterized. Both clones presented, at the amino acid level, an identity ranging from 74 to 85% with other NADH-GOGAT enzymes and a similarity lower than 46% with Fd-GOGAT enzymes already characterized from different plants. The analysis of the deduced amino acid sequence of each of the PvNADH-GOGATs showed an amido transferase domain between amino acids 96 and 454 (Figs 1 & 2). In addition, three regions involved in cofactor binding are also detected; namely, the sequences similar to those implicated in FMN binding (between the residues 1180 and 1253) (Fig. 1, underlined), which are adjacent to three conserved cysteine residues (1251, 1257 and 1262) (Fig. 1, diamonds) that are involved in binding the [3Fe-4S] cluster (Knaff et al. 1991; Sakakibara et al. 1991; Vanoni & Curti 2005), and a third domain containing five highly conserved amino acids (Fig. 1, arrowheads), which are critical for NADH binding (Scruton, Berry & Perham 1990; Suzuki & Knaff 2005). These regions in PvNADH-GOGATs are very similar and almost in the same positions as that described in MsNADH-GOGAT (Gregerson et al. 1993). Further, the amino acid sequence also exhibited a region (residues 1-98) encoding a transit peptide that predicts a plastid localization for both PvNADH-GOGATs. These data are in

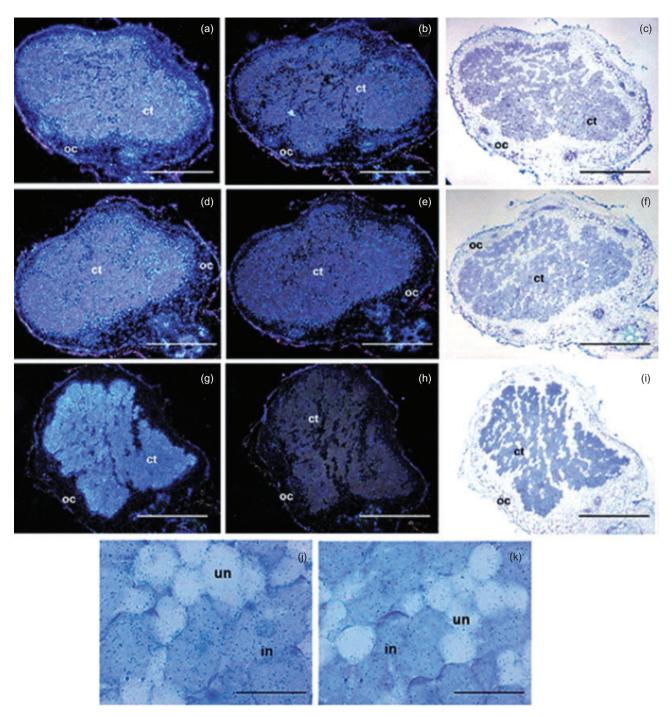


Figure 7. Localization of PvNADH-dependent glutamate synthase (NADH-GOGAT)-I and II transcripts in Phaseolus vulgaris root nodules by in situ hybridization. Longitudinal sections of paraffin embedded P vulgaris mature root nodules probed with sense and antisense ^{35}S UTP-labelled RNA probes. The signal specific to the radio-labelled probe is seen as white silver grains when tissue is viewed under dark field optics and as dark particles when viewed under bright field. (a and b) Nodule sections probed with PvNADH-GOGAT-II radio-labelled antisense (a) and sense (b) riboprobe. (d and e) Nodule sections probed with PvNADH-GOGAT-II radio-labelled antisense (d) and sense (e) riboprobe. (g and h) Nodule sections probed with Pv-leghemoglobin radio-labelled antisense (g) and sense (h) riboprobe. Panels (c, f and i) are bright field views of (a, d and g) to show nodule anatomy. (j and k) High magnification of portions of the panels (a) and (d), respectively, that show signal (dark grains) located predominately in the infected cells of the central tissue. Magnification for (a-i) = 40x, (j and k) = 400x. Bar length equals 0.5 mm (a-i), 0.05 mm (j and k). ct, central tissue; oc, outer cortex; in, infected cell; un, uninfected cell.

concordance with the previous results, which indicated that in bean nodules, the two NADH-GOGAT isoenzymes are localized in the plastids (Chen & Cullimore 1989). In contrast to the above, however, the amino acid analysis also revealed a region that distinguishes PvNADH-GOGATs from each other. A sequence comprising of 26 amino acids in the amido transferase domain of PvNADH-GOGAT-I is found to be completely different from the corresponding sequence in PvNADH-GOGAT-II (Fig. 2, designated in bold letters). This dissimilarity in the amino acid composition at the amido transferase domain between both PvNADH-GOGATs could explain the different affinities for L-glutamine between the two NADH-GOGAT isoenzymes reported by Chen & Cullimore (1988).

The data presented here demonstrate that contrary to what has been reported for other plants, in P. vulgaris, NADH-GOGAT is encoded by two different genes, and strongly suggest that these genes encode the two NADH-GOGAT isoenzymes reported by Chen & Cullimore (1988). The fact that the PvNADH-GOGAT-II, as compared to PvNADH-GOGAT-I, is strongly expressed in nodules (present study; Figs 4a,b & 6f,i,o,r) and the NADH-GOGAT-II isoenzyme, in contrast to NADH-GOGAT-I isoform, accounted for most of the nodule GOGAT activity (Chen & Cullimore 1988), will allow us to suggest that the PvNADH-GOGAT-II gene most likely encodes the NADH-GOGAT-II isoenzyme, while the PvNADH-GOGAT-I gene codes for the NADH-GOGAT-I isoenzyme. This conclusion gains further support from the finding that the expression of NADH-GOGAT-I, but not NADH-GOGAT-II, is affected by nitrogen fixation in the nodules (Fig. 4a,b), as was similarly reported for NADH-GOGAT-I and II isoenzyme activities in response to nitrogen fixation (Chen et al. 1990).

The interaction between *cis*-regulatory elements present in a promoter region and transacting factors is an indispensable requirement for regulating gene expression. Specific cis-regulatory elements in the promoter are probably shared by the genes that are responsive to similar cellular cues/factors. In silico analysis against the plant regulatory element databases revealed the presence of cis sequences potentially responsible for organ-/tissue-specific, and sugar responsive expression in the promoters of both PvNADH-GOGAT-I and -II (Fig. 8). The analysis showed that even though both promoters contained meristematic and vascular tissue-specific regulatory motifs, PvNADH-GOGAT-I promoter harboured more of such cis-elements, both in terms of number and diversity, than PrPvNADH-GOGAT-II (Fig. 8a,b). In contrast, PvNADH-GOGAT-II contained higher concentration of nodule-specific ciselements in its promoter (Fig. 8c). In consonance with these observations, we found a relatively higher activity of PvNADH-GOGAT-I promoter in the meristematic tissues (particularly lateral root primordia), as well as in the vascular traces (Fig. 6a-d), while PvNADH-GOGAT-II promoter exhibited greater activity in the nodules (Fig. 6q,r). In both promoters, we have also located several sugar responsive plant cis-acting elements (Fig. 8d,e) which

perhaps participate in the sucrose-mediated regulation of the expression of both these NADH-GOGAT genes as well (Fig. 5a). RT-PCR analyses showed that the PvNADH-GOGAT-I expression, but not that of PvNADH-GOGAT-II, is regulated by glutamine and asparagine (Fig. 5b). Searches of plant regulatory element databases did not present any records of cis-elements that mediate amino acid responsive expression of plant promoters. On the other hand, the examination of the promoter sequences of both PvNADH-GOGAT genes against animal genome sequences revealed the presence of consensus sequences resembling amino acid responsive cis-elements encountered in mammalian asparagine synthetase and CHOP gene promoters (Fig. 8f). In the mammalian asparagine synthetase gene, the palindromic sequence CATGATG localized in the proximal portion of the promoter region was found to be necessary for the regulation by amino acid availability (Guerrini et al. 1993). In our studies, the PvNADH-GOGAT-I promoter was also found to carry the palindromic sequence CATGATG in its proximal region. Future work dealing with deletion and mutational analyses of the NADH-GOGAT promoters can only clarify the precise significance and function of the above described potential cis-acting elements in the regulation of tissuespecific, and sugar and amino acid responsive expression of these genes in bean roots.

As compared to PvNADH-GOGAT-II, relatively low expression of PvNADH-GOGAT-I in nodules (Figs 4 & 6i) and its down regulation in the presence of amides and ureides (Fig. 5b) strongly suggest that this NADH-GOGAT isoform may not be playing a main role in the ammonia assimilation process in bean plant, which is a ureide transporter (Atkins 1982). However, a moderate enhancement in the expression of PvNADH-GOGAT-I in nodules at day 18 (Fig. 4) probably implies that this isoform may be performing a supportive role in ammonia assimilation during the active phase of nitrogen fixation. In contrast, high levels of expression of PvNADH-GOGAT-II in nodules (Fig. 4), particularly in the nitrogen-fixing infected cells (Figs 6r & 7d), and the maintenance of elevated levels of expression even in the presence of excessive concentrations of amides and ureides (Fig. 5b) point to the possibility that PvNADH-GOGAT-II may be the main isoform that mediates ammonia assimilation. The differential susceptibility for expression of these two NADH-GOGATs to various nitrogen compounds could be an adaptative mechanism allowing the N₂-fixing bean plants to mobilize ammonia, derived from nitrogenase activity, efficiently in the form of ureides (Atkins 1982) instead of amides.

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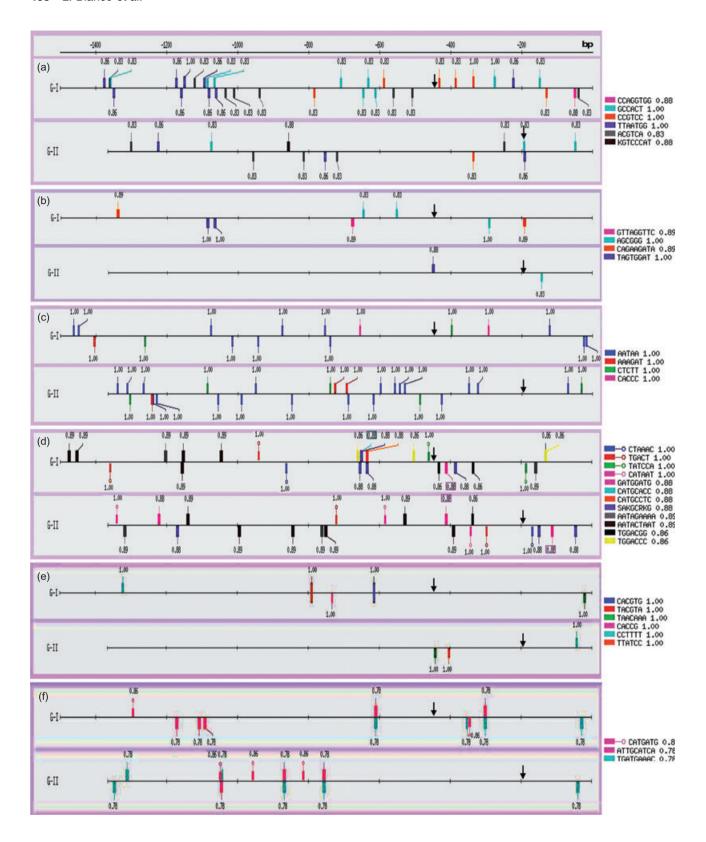


Figure 8. Diagram depicting the occurrence and location of various putative motifs within PvNADH-dependent glutamate synthase (NADH-GOGAT)-I and -II promoters that mediate organ-/tissue-specific, and sugar and amino acid responsive expression. Consensus sequences for (a) meristem-specific expression; CCAGGTGG (Kosugi, Suzuka & Ohashi 1995), GCCACT, CCGTCC (Meshi, Taoka & Iwabuchi 2000), TTAATGG (Kamiya et al. 2003), ACGTCA (Terada et al. 1995) and KGTCCCAT (Kosugi et al. 1995; Kosugi & Ohashi 1997; Klinedinst et al. 2000); (b) vascular expression: GTTAGGTTC (Patzlaff et al. 2003), AGCGGG (Lacombe et al. 2000), CAGAAGATA (Yin et al. 1997) and TAGTGGAT (Elliott & Shirsat 1998; Nontachaiyapoom et al. 2007); (c) nodule-specific expression: AATAA, AAAGAT, CTCTT and CACCC (Sandal, Bojsen & Marcker 1987; Stougaard et al. 1990; Szczyglowski et al. 1994; Vieweg et al. 2004; Fehlberg et al. 2005); (d) sugar-inducible expression: CTAAAC (Zourelidou et al. 2002), TGACT (Sun et al. 2003), TATCCA (Lu, Lim & Yu 1998; Lu et al. 2002), CATAAT (Li et al. 2006), GATGGATG, CATGCACC, CATGCCTC, SAKGCRKG (Geisler, Kleczkowski & Karpinski 2006), AATAGAAAA (Sun et al. 2003), AATACTAAT (Grierson et al. 1994), TGGACGG and TGGACCC (Maeo et al. 2001; Morikami et al. 2005); (e) sugar repression: CACGTG (Menkens, Schindler & Cashmore 1995; Urwin & Jenkins 1997), TACGTA (Toyofuku, Umemura & Yamaguchi 1998), CACCG (Niu, Helentjaris & Bate 2002), TAACAAA, CCTTTT (Morita et al. 1998) and TTATCC (Tatematsu et al. 2005); (f) amino acid responsive expression: CATGATG (Guerrini et al. 1993), ATTGCATCA (Bruhat et al. 2000) and TGATGAAAC (Barbosa-Tessmann et al. 2000; Bruhat et al. 2002). Numbers following the motif sequences depict the degree of conservation of the cis-elements (1.00 depicts the perfect match). Actual cis-element sequences, together with the highest degree of conservation of a given motif, are represented on the right side of the panels.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Figure S1. Sequences of the *Phaseolus vulgaris NADH-dependent glutamate synthase* (*NADH-GOGAT*)-*I* and *NADH-GOGAT-II* 5' flanking regions. The upstream 1501 bp and 1366 bp flanking regions of the *NADH-GOGAT-I* and *NADH-GOGAT-II* genes, respectively, from the putative translation start codons are shown. TATA box is highlighted in red, and CAAT boxes in green, and predicted transcription start site is shaded in blue. Uppercase letters denoted in black correspond to the 1065 bp and 1174 bp regions of the putative *NADH-GOGAT-II* and *NADH-GOGAT-II* promoters, whereas the letters indicated in blue correspond to the 436 bp and 192 bp 5' untranslated regions of the respective cDNAs. The first nucleotide upstream of the *NADH-GOGAT-II* and *NADH-GOGAT-II* ORFs is labelled as –1.

Table S1. Profiles of PCR cycles employed for isolating full-length *PvNADH-dependent glutamate synthase* (*NADH-GOGAT*)-*I* and -II cDNAs, and RT-PCR

Table S2. Primers used for obtaining the 5' regions of *PvNADH-dependent glutamate synthase* (*NADH-GOGAT*)-*I* and -*II* full-length cDNAs, and RT-PCR analysis.

Table S3. PCR primers used for genome walking of the 5' flanking region and amplification of the full-length promoters of *PvNADH-dependent glutamate synthase* (*NADH-GOGAT*)-*I* and -*II* genes.

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